# PCT

## INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

(Chapter II of the Patent Cooperation Treaty)

(PCT Article 36 and Rule 70)

| Applicant's or agent's file reference<br>F18754 GSK | **FOR FURTHER ACTION** | See Form PCT/IPEA/416 |
|---|---|---|
| International application No.<br>PCT/IB2005/000192 | International filing date *(day/month/year)*<br>27.01.2005 | Priority date *(day/month/year)*<br>28.01.2004 |
| International Patent Classification (IPC) or national classification and IPC<br>INV. C12N9/96 C12N9/20 | | |
| Applicant<br>CSIR et al. | | |

1. This report is the international preliminary examination report, established by this International Preliminary Examining Authority under Article 35 and transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of 5 sheets, including this cover sheet.
3. This report is also accompanied by ANNEXES, comprising:
   a. ☒ *sent to the applicant and to the International Bureau)* a total of 5 sheets, as follows:
      ☒ sheets of the description, claims and/or drawings which have been amended and are the basis of this report and/or sheets containing rectifications authorized by this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions).
      ☐ sheets which supersede earlier sheets, but which this Authority considers contain an amendment that goes beyond the disclosure in the international application as filed, as indicated in item 4 of Box No. I and the Supplemental Box.
   b. ☐ *(sent to the International Bureau only)* a total of (indicate type and number of electronic carrier(s)) , containing a sequence listing and/or tables related thereto, in celectronic form only, as indicated in the Supplemental Box Relating to Sequence Listing (see Section 802 of the Administrative Instructions).

4. This report contains indications relating to the following items:

| | | |
|---|---|---|
| ☒ | Box No. I | Basis of the report |
| ☐ | Box No. II | Priority |
| ☐ | Box No. III | Non-establishment of opinion with regard to novelty, inventive step and industrial applicability |
| ☐ | Box No. IV | Lack of unity of invention |
| ☒ | Box No. V | Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement |
| ☐ | Box No. VI | Certain documents cited |
| ☐ | Box No. VII | Certain defects in the international application |
| ☐ | Box No. VIII | Certain observations on the international application |

| Date of submission of the demand | Date of completion of this report |
|---|---|
| 11.11.2005 | 24.04.2006 |
| Name and mailing address of the international preliminary examining authority:<br>European Patent Office<br>D-80298 Munich<br>Tel. +49 89 2399 - 0 Tx: 523656 epmu d<br>Fax: +49 89 2399 - 4465 | Authorized officer<br>Valcarcel, R<br>Telephone No. +49 89 2399-2368 |

Form PCT/IPEA/409 (Cover Sheet) (April 2005)

**Box No. I Basis of the report**

1. With regard to the **language**, this report is based on the international application in the language in which it was filed, unless otherwise indicated under this item.

   ☐ This report is based on translations from the original language into the following language , which is the language of a translation furnished for the purposes of:
   - ☐ international search (under Rules 12.3 and 23.1(b))
   - ☐ publication of the international application (under Rule 12.4)
   - ☐ international preliminary examination (under Rules 55.2 and/or 55.3)

2. With regard to the **elements*** of the international application, this report is based on *(replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report)*:

| | |
|---|---|
| **Description, Pages** | |
| 1-16 | as originally filed |
| **Claims, Numbers** | |
| 1-29 | filed with the demand |
| **Drawings, Sheets** | |
| 1/4-4/4 | as originally filed |

   ☐ a sequence listing and/or any related table(s) - see Supplemental Box Relating to Sequence Listing

3. ☐ The amendments have resulted in the cancellation of:
   - ☐ the description, pages
   - ☐ the claims, Nos.
   - ☐ the drawings, sheets/figs
   - ☐ the sequence listing *(specify)*:
   - ☐ any table(s) related to sequence listing *(specify)*:

4. ☐ This report has been established as if (some of) the amendments annexed to this report and listed below had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).
   - ☐ the description, pages
   - ☐ the claims, Nos.
   - ☐ the drawings, sheets/figs
   - ☐ the sequence listing *(specify)*:
   - ☐ any table(s) related to sequence listing *(specify)*:

* *If item 4 applies, some or all of these sheets may be marked "superseded."*

**Box No. V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

1. Statement

| | | | |
|---|---|---|---|
| Novelty (N) | Yes: | Claims | 1-29 |
| | No: | Claims | NONE |
| Inventive step (IS) | Yes: | Claims | 1-29 |
| | No: | Claims | NONE |
| Industrial applicability (IA) | Yes: | Claims | 1-29 |
| | No: | Claims | NONE |

2. Citations and explanations (Rule 70.7):

**see separate sheet**

Re Item V

1. The document numbering corresponds to the order of citation in the International Search Report.

2. The subject-matter of claims 1-29 is new in the sense of Article 33(2) PCT.

   D1 discloses a process for producing an enzyme (glucose oxidase) preparation, wherein an aqueous solution comprising an enzyme is emulsified with an hydrophobic phase (see claim 5, e.g. a perfluoropolyalkylether synthetic oil, and see column 10, lines 20-23), and treated with a crosslinker (see claim 1, e.g. glutaraldehyde, see column 10, line 59), so that the enzyme is crosslinked (see claim 1).

   Thus, the emulsion of the enzyme and the perfluorocarbon liquid is stabilized by chemical crosslinking of the enzyme to form a gel. This gel is used to for the immobilization of enzymes in a detector for glucose determination (see abstract).

   However, in D1 there are apparently no enzyme particles formed but rather a continuous gel. The particles which are disclosed in D1 are the particles of the material that dissolve the oxygen and not enzyme particles (see column 8, lines 56-59).

   Furthermore, the method of D1 does not include an step of recovering the enzyme particles from the hydrophobic (O) phase. Thus, the subject-matter of claims 1-29 is novel over D1.

   It is noted that in the methods of D1, the oil phase content used is between 5% and 20% by volume (column 9, lines 43-45). It appears that at such low oil concentration only oil in water (O/W) emulsions would be formed, and not water in oil (W/O) emulsions as it is the case under the process defined in claim 1 of the present application.

3. Claims 1-29 meet the criteria of Article 33(3) PCT. D3 is regarded as being the closest prior art to the subject-matter of claim 1 since it relates to the same field as the present application, the generation of enzyme particles (in particular lipase particles) which can be used as catalysts. D3 discloses cross-linked enzyme aggregates (CLEAs) by precipitating lipases with different agents and by chemical cross-linking of the enzyme

(see abstract). D3 also disclose cross-linked enzyme crystals (CLECs) which are highly active and stable biocatalysts (see page 1379, left column, first paragraph).

The subject-matter of claim 1 differs from the process of D3 in that in D3 there is no water in oil (W/O) emulsion step before cross-linking the enzyme molecules, and therefore, also there is not recovery from the second liquid phase.

The problem to be solved by the present invention may be regarded as to provide an alternative process for producing stabilized enzyme particles suitable for use as a catalyst. The solution to this problem proposed in claim 1 of the present application is considered as involving an inventive step (Article 33(3) PCT) for the following reasons:

In both CLEC and CLEA, some active sties of the enzymes are not exposed (see e.g. figure 1 of D3). Thus, there was an incentive to provide alternative methods to provide enzyme particles.

Water-in-oil and water-in-oil-in-water emulsions for the preparation of enzyme microspheres for protein delivery were standard in the art (see e.g. abstract or figure 1 of D2; pages 53 and 54 of D4; or figure 4 of D5). However, it is considered that there was no motivation in the prior art to combine the teachings of D3 with the teaching of documents relating for protein delivery in order to add a emulsion step before the corsslinking as referred to in claim 1 of the present application. Thus, claim 1 of the present application is considered inventive.

3.1 Claim 24 refers to enzyme particles which (although not referring back to the method of claim 1) have the technical features of particles obtained by the method of claim 1. Thus, also claim 24 is considered inventive.

3.2 Claims 2-23, and 25-29 are defined in terms of claims 1 and 24 and as such also meet the requirements of the PCT with respect to novelty and inventive step.

CLAIMS:

1. A process for producing enzyme particles, which process includes

providing an emulsion of droplets of a first liquid phase dispersed in a second liquid phase, with the one liquid phase being a hydrophilic phase and the other liquid phase being a hydrophobic phase which is immiscible with the hydrophilic phase, and with enzyme molecules being located at or within interfacial boundaries of the droplets and the second liquid phase;

cross-linking the enzyme molecules of the respective droplets so that individual enzyme particles, which are stable and in which the enzymes are immobilized with a majority of active sites of the enzymes being orientated either internally or externally, are formed from individual droplets; and

recovering the individual enzyme particles from the second liquid phase.

2. A process according to Claim 1, wherein the individual particles have openings so that the liquid phases can pass in or out of the particles.

3. A process according to Claim 1, wherein individual particles are liquid impervious.

4. A process according to any one of Claims 1 to 3 inclusive, which includes adding to the hydrophilic phase and/or to the hydrophobic phase and/or to the emulsion, a modifier for modifying the hyrophobicity and/or charge of the enzyme.

5. A process according to any one of Claims 1 to 4 inclusive, wherein the enzyme is a lipase.

6. A process according to Claim 5, wherein the lipase is selected from Pseudomonas cepacia lipase, Pseudomonas fluorescens lipase, Pseudomonas alcaligenes lipase, Candida rugosa lipase, Candida antarctica lipase A, Candida antarctica lipase B, Candida utilis lipase, Thermomyces

lanuginosus lipase, Rhizomucor miehei lipase, Aspergillus niger lipase, Aspergillus oryzae lipase, Penicillium sp lipase, Mucor javanicus lipase, Mucor miehei lipase, Rhizopus arrhizus lipase, Rhizopus delemer lipase, Rhizopus japonicus lipase, Rhizopus niveus lipase, and Porcine Pancreatic lipase.

7. A process according to Claim 5 or Claim 6, wherein the provision of the emulsion is effected by dissolving the enzyme in the hydrophilic or W phase and forming the emulsion by mixing the enzyme containing hydrophilic phase with the hydrophobic or O phase.

8. A process according to Claim 7, which includes selectively precipitating the enzyme at the interface when the emulsion is a O/W emulsion in which hydrophobic phase droplets are dispersed in a continuous hydrophilic phase, or within the droplet volume, when the emulsion is a W/O emulsion in which hydrophilic phase droplets are dispersed in a continuous hydrophobic phase.

9. A process according to Claim 7 or Claim 8, wherein the cross-linking of the enzyme molecules is effected by means of a cross-linking agent which is added to the hydrophilic phase and/or to the hydrophobic phase and/or to the emulsion.

10. A process according to Claim 9, which includes adding to the hydrophilic phase and/or to the hydrophobic phase and/or to the emulsion, a temporary protectant that occupies active sites of the enzyme during the cross-linking, thereby inhibiting occupation of or reaction with the active sites by the cross-linking agent.

11. A process according to any one of Claims 7 to 10 inclusive, which includes adding an amino acid to the emulsion to inhibit agglomeration of the individual enzyme particles.

12. A process according to any one of Claims 7 to 11 inclusive, which includes recovering the enzyme particles from the second liquid phase.

13. A process according to any one of Claims 7 to 12 inclusive, which includes extracting the first liquid phase from the enzyme particles.

14. A process according to any one of Claims 7 to 13 inclusive, wherein the hydrophilic phase comprises water and, optionally, a buffer in the water.

15. A process according to any one of Claims 7 to 13 inclusive, wherein the hydrophilic phase comprises a polyethylene glycol and, optionally, water admixed with the polyethylene glycol.

16. A process according to any one of Claims 7 to 15 inclusive, wherein the hydrophobic phase comprises an oil; a hydrocarbon; an ether; or an ester.

17. A process according to any one of Claims 7 to 16 inclusive, wherein the emulsion is a W/O emulsion in which hydrophilic phase droplets are dispersed in a continuous hydrophobic phase, with a second enzyme, co factor and/or mediator being present in the hydrophilic phase.

18. A process according to any one of Claims 5 to 16 inclusive, wherein a triglyceride, which is hydrolysable by lipase, is used as the hydrophobic phase, with an O/W emulsion, in which hydrophobic phase droplets are dispersed in a continuous hydrophilic phase, being formed and with the dispersed hydrophobic phase contained within the cross-linked particles being hydrolyzed by the lipase during and after the cross-linking reaction.

19. A process according to any one of Claims 7 to 16 inclusive, wherein an initial O/W emulsion, in which hydrophobic phase droplets are dispersed in a continuous hydrophilic phase, is formed, with the process including, before effecting the cross-linking, centrifuging the emulsion and separating a concentrated emulsion from a dilute hydrophilic phase, to

increase lipase purity; and inverting the emulsion to form a W/O emulsion in which hydrophilic phase droplets are dispersed in a continuous hydrophobic phase, by the addition of a surfactant with a lower HLB value.

20. A process according to any one of Claims 1 to 19 inclusive wherein, to impart specific properties to the enzyme particles, a modifier is added to the hydrophilic phase and/or to the hydrophobic phase and/or to the emulsion.

21. A process according to Claim 20, wherein the modifier is a surfactant, for imparting enhanced enzyme activity and improved emulsion stability.

22. A process according to Claim 20, wherein the modifier is a precipitator for precipitating the enzyme onto the emulsion interfaces.

23. A process according to Claim 20, wherein the modifier is an additive for modifying the pH; ionic strength; viscosity; magnetic properties; agglomeration tendency; and/or zeta potential of the emulsion and/or the enzyme particles.

24. An enzyme particle, which comprises cross-linked enzyme molecules so that the particle is stable, with the particle being hollow, and in which the enzymes are immobilized, with a majority of active sites of the enzymes being orientated either internally or externally.

25. An enzyme particle according to Claim 24, which is spherical.

26. An enzyme particle according to Claim 24 or Claim 25, which contains, in its lumen, a liquid.

27. An enzyme particle according to any one of Claims 24 to 26 inclusive, wherein the enzyme is a lipase.

28. An enzyme particle according to Claim 27, wherein the lipase is selected from Pseudomonas cepacia lipase, Pseudomonas fluorescens lipase, Pseudomonas alcaligenes lipase, Candida rugosa lipase, Candida antarctica lipase A, Candida antarctica lipase B, Candida utilis lipase, Thermomyces lanuginosus lipase, Rhizomucor miehei lipase, Aspergillus niger lipase, Aspergillus oryzae lipase, Penicillium sp lipase, Mucor javanicus lipase, Mucor miehei lipase, Rhizopus arrhizus lipase, Rhizopus delemer lipase, Rhizopus japonicus lipase, Rhizopus niveus lipase, and Porcine Pancreatic lipase.

29. A method of carrying out a reaction, which includes allowing a reaction medium to undergo a reaction in the presence of a plurality of the enzyme particles according to any one of Claims 24 to 28 inclusive, with the reaction thus being catalyzed by the enzyme particles.